

ORIGINAL ARTICLE

Immunogenic properties of Landrace pigs selected for resistance to mycoplasma pneumonia of swine

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ABSTRACT

Mycoplasma pneumonia of swine (MPS) lung lesions and immunogenic properties were compared between a Landrace line that was genetically selected for reduced incidence of pulmonary MPS lesions, and a non-selected Landrace line. The MPS-selected Landrace line showed significantly lower degrees of pulmonary MPS lesions compared with the non-selected Landrace line. When changes in immunity before and after vaccination were compared, the percentage of B cells in the peripheral blood of the MPS-selected Landrace line was significantly lower than that of the non-selected line. Furthermore, the concentration of growth hormone and the mitogen activity of peripheral blood mononuclear cells in the MPS-selected Landrace line showed significantly ($P < 0.05$) lower increases after vaccination than the non-selected line. Conversely, the concentration of peripheral blood interferon (IFN)- γ and salivary immunoglobulin A (IgA) after *Mycoplasma hyopneumoniae* vaccination was significantly higher in the MPS-selected Landrace line than in the non-selected line. Gene expression of toll-like receptor (TLR)2 and TLR4 was significantly higher in the MPS-selected Landrace line in immune tissues, with the exception of the hilar lymph nodes. The present results suggest that peripheral blood IFN- γ , salivary IgA, TLR2, and TLR4 are important immunological factors influencing the development of MPS lesions.

Key words: disease resistance, immunogenic properties, mycoplasma pneumonia lesions, pig.

INTRODUCTION

Mycoplasma pneumonia of swine (MPS) is a global endemic chronic respiratory disease in the swine industry. This disease, which is induced mainly by *Mycoplasma hyopneumoniae* infection, causes sizeable economic losses due to increased veterinary costs and product loss (Thacker *et al.* 1999; Maes *et al.* 2008). *M. hyopneumoniae* is located in the lumina of the respiratory tract, as well as the mucosal surface of the trachea, bronchi and bronchioles (Blanchard *et al.* 1992); it can propagate on tracheal and bronchial surfaces after infection. In MPS, the bronchial and bronchiolar epithelia show a loss of cilia from the epithelial cells and sloughing of epithelial cells into the airway lumina (Redondo *et al.* 2009). The primary clinical sign is a sporadic, dry, nonproductive cough (Thacker *et al.* 1999). Destruction of the cilia by *M. hyopneumoniae* increases the morbidity to other pathogens such as *Pasteurella multocida*, porcine respiratory virus, reproductive syndrome virus, porcine circovirus type 2, and porcine respiratory coronavirus (Ciprian *et al.* 1988; Thacker *et al.* 1999; Kim *et al.* 2003; Maes *et al.* 2008).

Several strategies for the control of infectious diseases in swine are commonly employed, such as improved housing conditions, use of antimicrobial agents and vaccination. However, these measures cannot completely eradicate the involved pathogens or inhibit their transmission. Further, the residue of the antibiotic in livestock products and antibacterial substances in domesticated animals is heavily regulated in Japan (The Ministry of Agriculture, Forestry and Fisheries, Japan 2014). Therefore, safer and more efficient ways to control swine chronic respiratory diseases are necessary.

From the point of view of genetic animal breeding, selection for disease resistance is very useful to ultimately minimize the need for medication. For example, Kadowaki *et al.* (2012) have improved the Landrace breed population by selecting for resistance to MPS and for greater meat production at the Miyagi Prefecture Animal

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Industry Experiment Station. Previously, we have reported the immune responsiveness of this MPS-selected Landrace line to sheep red blood cells (SRBCs) (Katayama *et al.* 2011) and *M. hyopneumoniae* vaccine sensitization (Shimazu *et al.* 2013, 2014). However, the relationship between MPS lung lesions and immune responsiveness has not been reported. Therefore, the present study compared the MPS disease resistance of MPS-selected Landrace pigs and non-selected Landrace pigs by assessing their lung lesions and immunogenic characteristics.

MATERIALS AND METHODS

Experimental animals

All experimental animals were maintained in a conventional pig barn at Miyagi University School of Food, Agricultural and Environmental Sciences. To confirm repeatability, two experiments were conducted. In experiment 1, we used 12 castrated Landrace pigs (average age, 16 weeks), which were selected for lower levels of MPS lesions for five generations at the Miyagi Prefecture Animal Industry Experiment Station (Kadowaki *et al.* 2012), and 12 non-selected castrated Landrace pigs from the Research and Development Center, Nippon Meat Packers, Inc., Hokkaido, Japan. In experiment 2, we used six MPS-selected (as describe above) castrated Landrace pigs (average 17 weeks of age), and six non-selected castrated Landrace pigs from the Fukushima Livestock Experiment Station.

All experimental animal procedures were approved by Tohoku University and Miyagi University regulations concerning the protection and care of experimental animals.

Experimental design

In experiment 2 as well as experiment 1, animals of each line were bred in the same group and allowed to acclimatize for about 1 month before the experiment began. Pigs were raised in a crude, vinyl-roofed, open-air barn with a bedding of rice husks. The bedding was neither changed or cleaned, nor was the excrement removed during the experiment, in order to provide stressful conditions with a bad environment. During the experimental period, *M. hyopneumoniae* vaccine (HYORESP[®]; Merial Japan Ltd., Tokyo, Japan), which consisted of an inactivated, adjuvant whole-cell preparation, was injected intramuscularly twice, once on day -7 and again, on day 0, after blood collection. Thus, day 0 was defined as the day when the second dose of vaccine was administered. Immune functional analyses of the samples were conducted on days -7, 0, 2, 7 and 14. Blood samples were collected via jugular venipuncture into vacuum tubes containing heparin and ethylenediaminetetraacetic acid (EDTA; Terumo, Tokyo, Japan). At each sampling time, salivary samples were collected from the oral cavity with a cotton swab. In order to confirm the presence of pathogenic *M. hyopneumoniae*, we sampled the nasal mucus of each animal at each blood sampling time. On day 14, all pigs were killed by stunning at the anatomy facilities of Tohoku University, and immune tissues were collected. At this time, pulmonary MPS lesions were confirmed and measured by the veterinarian. In experiment 1, half of the pigs (six MPS-selected and six non-selected) were killed on day 14, and the remaining pigs remained until they reached a body weight of approximately 110 kg (average age, 23 weeks), when they were transported to the slaughterhouse (day 30 in this experimental period) to be slaughtered at the Miyagi Prefecture meat market center. After slaughter, pulmonary MPS lesions were examined. Blood samples were not collected from these animals on day 14.

Examination of pulmonary MPS lesions

After slaughter, the pulmonary MPS lesions were confirmed and scored according to Goodwin's lung score (Goodwin 1972) by a trained veterinarian.

Analysis of immune cells

The total number of white blood cells (WBCs) in 1 μ L of peripheral blood was measured using an autohemolytic counter (Celltec; Nihon Kodan, Tokyo, Japan). The percentage and number of each type of immune cell were measured by flow cytometry assay. Heparin-treated swine blood was mixed with 2% dextran-phosphate-buffered saline (PBS) to allow for erythrocyte sedimentation and blood leukocyte enrichment into the supernatant (Genovesi *et al.* 1989; Yang & Parkhouse 1996). The upper phase was retained, and the cells were washed before a brief resuspension in hypotonic ammonium chloride solution to remove erythrocytes. Cells were stained with fluorescent antibodies in PBS containing 2% (v/v) fetal bovine serum (FBS) and 0.01% (w/v) sodium azide, and subjected to flow cytometry analysis. Cells were labeled with the following primary antibodies: biotin conjugated-CD3 ϵ (PPT₃), phycoerythrin (PE)-CD8 α (73-21-11), fluorescein isothiocyanate (FITC)-CD4 α (74-12-4), FITC-SWC3a (CD172a: 74-22-15), PE-CD21 (BB6-11C9.6), and unconjugated CD25 (K23.3B2, mouse immunoglobulin G1 (IgG1); AbD Serotec, Oxford, UK). The binding of unlabeled CD25 monoclonal antibody and biotin-conjugated CD3 ϵ was visualized using phycoerythrin (PE)-goat F(ab')₂ anti-mouse IgG1 and streptavidin PE/Cy5 (Bio Legend, San Diego, CA, USA), respectively. All antibodies were obtained from Beckman Coulter (Fullerton, CA, USA), unless otherwise stated. The stained cells were analyzed using a FACSCalibur[™] fluorescence-activated cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) equipped with CellQuest software. Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

M. hyopneumoniae-specific IgG

Blood samples were collected from the cervical veins of the animals. The serum was separated by centrifugation (1600 \times g for 10 min at 4°C), divided into aliquots, and stored at -20°C until analysis. A 96-well *M. hyopneumoniae*-specific plate was incubated with *M. hyopneumoniae* vaccine (HYORESP[®]; Merial Japan Ltd., Tokyo, Japan) for 1 h at room temperature. Nonspecific binding of the antigen or antibody to the plate was blocked by treatment with Block Ace (UK-B40; DS Pharma Biomedical Co., Ltd., Osaka, Japan) for 2 h at room temperature. A 200-fold dilution of swine serum was then exposed to the plate; pooled swine serum was used as a standard (incubated for 2 h at 30°C). After washing with Tris buffered saline (T6664; Sigma, Tokyo, Japan), alkaline phosphatase-labeled Rabbit-Poly Anti-Pig IgG (H+L) Antigen (Rockland Immunochemicals Inc., Philadelphia, PA, USA) was added, and the samples were incubated for 24 h at 5°C to detect *M. hyopneumoniae*-specific IgG. After washing to remove nonspecific binding, fluorescence intensity was measured at 405 nm at 5-min intervals after the reaction with the enzyme substrate solution.

Salivary enzyme-linked immunosorbent assay (IgA)

Salivary samples were collected from the oral cavity of animals by using cotton swabs. The saliva was separated by centrifugation (at 1600 \times g for 10 min at 4°C), divided into sterilized microtubes (WATSON CO., LTD., Tokyo, Japan), and stored at -80°C until

analysis. A 96-well plate was incubated with affinity-purified goat anti-pig IgA coating antibody (A100-102A; Bethyl Laboratories, Inc., Montgomery, TX, USA) for 1 h at room temperature. Nonspecific binding of the antigen or antibody to the plate was blocked by treatment with Postcoat Solution (Bethyl Laboratories, Inc.) for 0.5 h at room temperature. A 500-fold dilution of swine saliva was then exposed to the plate. Pig reference serum (RS10-107-3) was used as a standard (incubated for 1 h at room temperature). After washing with Tris buffered saline, horseradish peroxidase (HRP)-conjugated goat anti-pig IgA detection antibody (A100-102P) was added, and the samples were incubated for 1 h at room temperature to detect the IgA. After 15 min of incubation with the enzyme substrate solution, the reaction was stopped by a stop solution, and the fluorescence intensity was measured at 450 nm.

Mitogenicity assay

Peripheral blood samples from day -7 (nonstimulated) and day 0 (7 days after the first *M. hyopneumoniae* vaccination) were used for this assay. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation from individual porcine blood samples and washed with PBS and Roswell Park Memorial Institute 1640 medium (Sigma, Tokyo, Japan), supplemented with 2% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin. Next, these cells were placed in a 96-well microplate (Corning Costar Japan, Tokyo, Japan; 2×10^5 cells/well) and stimulated with *M. hyopneumoniae* (2×10^6 cells/well; *M. hyopneumoniae* J (ATCC 25934), a non-pathogenic strain inactivated with formalin), lipopolysaccharide (LPS; 20 µg/well LPS from *Escherichia coli* O55:B5 prepared using phenol extraction followed by gel-filtration chromatography; Sigma), or concanavalin A (ConA; 2 µg/well). The cells were then maintained in an atmosphere containing 5% CO₂ at 37°C. After a 32-h incubation, the cells were radiolabeled with 9.25 kBq/well methyl-³H-thymidine (GE Healthcare, Tokyo, Japan) and incubated for a further 16 h. The cells were then harvested with a glass fiber filter (PerkinElmer Japan, Kanagawa, Japan). The methyl-³H-thymidine incorporation was counted in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA, USA). Subsequent results, presented as stimulation index (SI), were calculated using the following equation:

$$\frac{(\text{counts per minute in treated cultures}) - (\text{counts per minute in background})}{(\text{counts per minute in control cultures}) - (\text{counts per minute in background})}$$

For a comparison of SI before and after vaccination samples (relative SI), the following equation was applied:

$$\text{Relative SI} = \frac{(\text{SI from day 0 [after vaccination]})}{(\text{SI from day - 7 [before vaccination]})}$$

Cytokines in peripheral blood by ELISA and messenger RNA (mRNA) expression analysis by real-time PCR

Blood samples were collected from the cervical veins of the animals. The serum was separated by centrifugation (1600 × g for 10 min at 4°C), divided into aliquots, and stored at -20°C until analysis. The concentrations of interleukin (IL)-10, IL-13, IL-17, interferon (IFN)-γ and tumor necrosis factor (TNF)-α in serum samples were determined using commercially available ELISA kits according to the manufacturers' instructions (IL-10, IFN-γ and TNF-α, Invitrogen, Carlsbad, CA, USA and

IL-13 and IL-17, Bethyl Laboratories). Procedure of real-time quantitative PCR analysis to expression levels of mRNAs in blood was previously described by Shimazu *et al.* (2014).

Analysis of toll-like receptor (TLR) and cytokine mRNA expression by real-time PCR

In experiment 2, the expression levels of mRNAs from total blood leukocytes, and from immune tissues (Peyer's patch (Pp), the mesenteric lymph node (MLN), the hilar lymph node (HLN), the spleen, and pulmonary alveolar macrophages (PAMs)) were analyzed by real-time quantitative PCR. EDTA-treated peripheral blood samples (250 µL) were mixed with 750 µL of TRIzol LS Reagent (Invitrogen, Carlsbad, CA, USA) for total RNA extraction. PAMs were collected from the bronchoalveolar lavage fluid by centrifugation. Total RNA in PAMs and immune tissues was extracted by mixing with 1 ml of TRIzol Reagent and crushing with beads. The total RNA concentration extracted from each sample was measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and 1 µg of total RNA was subjected to reverse transcription. The complementary DNAs (cDNAs) were synthesized using a PrimeScript RT reagent kit with genomic DNA (gDNA) Eraser (Takara, Tokyo, Japan), according to the manufacturer's recommendations. Real-time quantitative PCR was performed using a Thermal Cycler Dice® Real Time System II (Takara) and SYBR® Premix Ex Taq II (Tli RNaseH Plus; Takara). The conditions for PCR amplification were as follows: 30 s at 95°C, followed by 40 cycles of 5 s each at 95°C, and finally, 30 s at 60°C. Expression of β-actin was used to normalize for the variations in total cDNA levels from sample to sample. The primer sequences used were as previously described (Moue *et al.* 2008).

Growth hormone analysis

Plasma concentrations of growth hormone (GH) were measured by double-antibody radioimmunoassay. Porcine GH (AFP-10888C; National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA) was used as the radio-iodinated antigen and standard. Porcine GH antiserum (AFP-422801; National Hormone and Peptide Program) was used at a dilution of 1:6 × 10⁵; the detection limit was 0.0361 ng/tube. The intra-assay coefficient of variation for GH was < 10%.

Statistical analysis

Data was analyzed using the SAS MIXED procedure (SAS Cary, NC, USA). The fixed effects were the pig lines (selected Landrace and non-selected Landrace) and the day of the experiment; the random effect was the animals. We compared the mean change in these two lines "after" vaccination. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Some results of experiment 1 have been previously reported (Shimazu *et al.* 2013, 2014). Therefore, to confirm the reproducibility of the immunity traits that had been reported by experiment 1, the results obtained in experiment 2 are mainly described.

There was no significant difference in the average daily gain of MPS-selected Landrace and non-selected Landrace lines in experiments 1 and 2 (data not shown).

Lung lesion scores

First, we confirmed *M. hyopneumoniae* DNA in nasal swabs from all pigs used in the two experiments (data not shown), confirming that this bacterium is indigenous in pigs. In experiment 1 and experiment 2, we found that MPS lung lesion scores were significantly lower in the MPS-selected Landrace line than in the non-selected Landrace line (Fig. 1). Two non-selected Landrace lines were introduced from a different farm, with a different genetic background and breeding environment. The MPS-selected line also showed

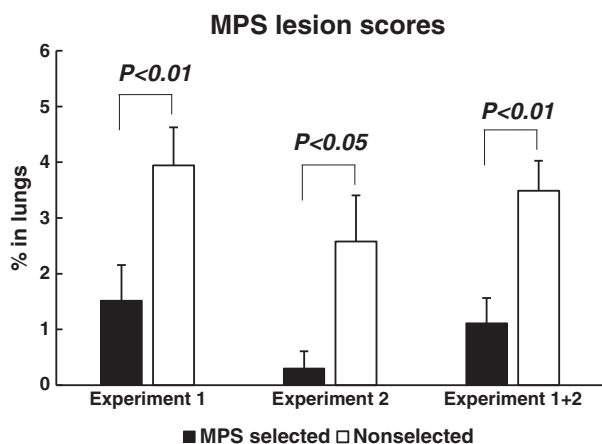


Figure 1 The pulmonary mycoplasma pneumoniae of swine (MPS) lesions of lungs from MPS-selected (black bars) and non-selected Landrace pigs (white bars) were confirmed by slaughter, and the scores were measured following Goodwin's lung score (1972). The results of experiment 1, experiment 2, and integrate of 1 and 2 are shown from left to right, respectively. Statistical differences are indicated as the comparison between the two lines on the same day. Error bars indicate the standard error.

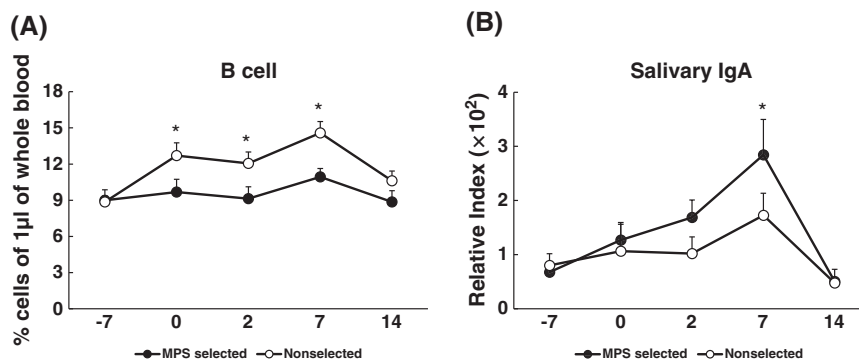


Figure 2 The average percent of B cells in 1 µL of peripheral blood, and the concentration of salivary immunoglobulin A (IgA) obtained from mycoplasma pneumoniae of swine (MPS)-selected ● (closed circles) and non-selected ○ (open circles) Landrace pigs. Blood samples were collected on 5 days (–7, 0, 2, 7 and 14). MPS vaccination was conducted on days –7 and 0 after blood sampling on those days. Statistical differences are indicated as the comparison between the two lines on the same day. Error bars indicate the standard error. (Asterisk mark indicates a significant difference at $P < 0.05$).

significantly lower lung lesion scores than either of these two non-selected lines.

Analysis of immune cells in peripheral blood

In experiment 2, there was no significant difference in the number of WBCs in 1 µL of the peripheral blood between MPS-selected Landrace and non-selected Landrace lines (data not shown). However, the percentage of B cells decreased as in the previous result (experiment 1, Shimazu *et al.* 2013) after *M. hyopneumoniae* vaccination, and was significantly higher in the non-selected Landrace line than in the MPS-selected Landrace line on days 2 and 7 after vaccination (Fig. 2A). We did not observe any significant difference in the numbers of any other immune cells between the MPS-selected Landrace and non-selected Landrace lines.

M. hyopneumoniae-specific IgG production and salivary IgA production

Although there was no significant difference in *M. hyopneumoniae* vaccine-specific IgG production in peripheral blood from the MPS-selected line and non-selected line in experiment 1 (Shimazu *et al.* 2014), in experiment 2, IgG tended to be more suppressed in the MPS-selected line than in the non-selected line (data not shown). In addition, in experiment 2, the IgA concentration in salivary glands 7 days after the second vaccination increased in both lines, and the increase was significantly greater in the MPS-selected line than in the non-selected line (Fig. 2B).

Mitogenicity assay

Cell proliferation activity between the MPS-selected and non-selected Landrace lines before and after vaccination was compared. In preset studies, we stimulated PBMCs harvested from these two lines with *M. hyopneumoniae*, LPS, and ConA to examine their proliferative abilities.

In the present study (experiment 2), the non-selected line showed significantly higher SI values stimulation with *M. hyopneumoniae* before and after the MPS vaccination, and with LPS before the MPS vaccination (Fig. 3A,B). However, no statistical difference for relative mitogen activity, which is calculated by dividing the SI value after vaccination by the SI value before vaccination, was observed (Fig. 3C).

The mRNA expression and concentration of cytokines in peripheral blood

In this study, the IFN- γ mRNA expression was significantly increased in the MPS-selected line at days 0 and 7 after vaccination compared with the non-selected line (Fig. 4). No significant difference in the mRNA expressions of any other cytokines between the MPS-selected Landrace and non-selected Landrace lines were observed (data not shown). The concentration of IFN- γ measured was not possible to show; because measurements by the ELISA kit indicated the value below the detection limit.

TLR and cytokine mRNA expression in immune tissues

In experiment 2, mRNA expression of TLR2 and TLR4 in Pps, MLNs and spleens, and TLR4 in PAMs, were significantly higher in the MPS-selected Landrace line than in

the non-selected line. However, there were no significant differences in the mRNA expression levels of TLR2 and TLR4 in HLNs, or that of TLR2 in PAMs, between

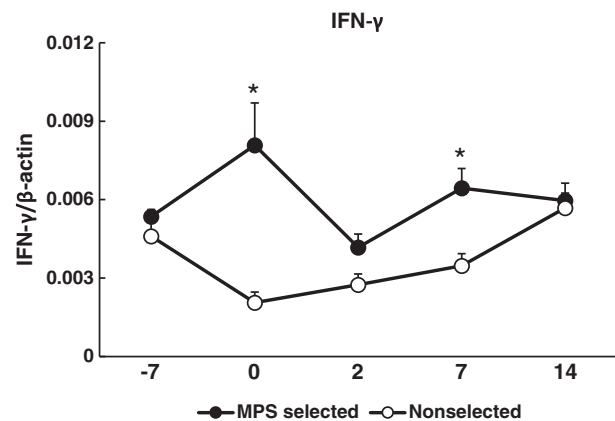


Figure 4 Mean and SE of the gene expression of interferon (IFN)- γ in peripheral blood from mycoplasma pneumoniae of swine (MPS)-selected \bullet (closed circles) and non-selected Landrace \circ (open circles) pigs. Blood samples were collected on 5 days ($-7, 0, 2, 7$ and 14). MPS vaccination was conducted on days -7 and 0 after blood sampling on those days. Statistical differences are indicated as the comparison between the two lines on the same day. The expression of IFN- γ is shown as value of relative index (RI), which is calculated by dividing the expression levels of IFN- γ by the expression levels of β -actin. (Asterisk mark indicates a significant difference at $P < 0.05$).

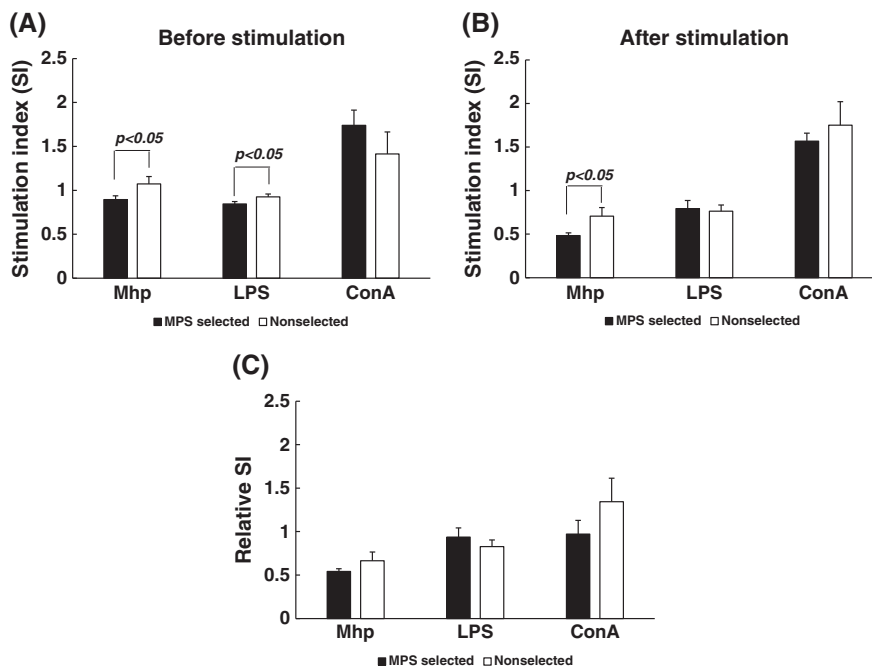


Figure 3 Mitogen activity of peripheral blood mononuclear cells (PBMCs) from mycoplasma pneumoniae of swine (MPS)-selected (black bars) and non-selected Landrace pigs (white bars). Mitogen activity was analyzed in PBMCs obtained from the two lines before and after sensitization with MPS vaccine. These cells were stimulated with *Mycoplasma hyopneumoniae* (2×10^6 cells/well), or lipopolysaccharide (LPS; $20 \mu\text{g/well}$), or concanavalin A (ConA; $2 \mu\text{g/well}$). The activities before (A) and after vaccination (B) are shown as values of stimulation index (SI). Relative SI, which is calculated by dividing the SI value after vaccination by the value before vaccination, is also shown (C).

MPS-selected and non-selected lines. On the other hand, the mRNA expressions of IL-6 in Pps and MLNs, and IL-10 in spleens, Pps and MLNs were significantly higher in the MPS-selected line than in the non-selected line. There were no significant differences in expression of IL-6 in the spleens, HLNs and PAMs, or IL10 in the HLNs and PAMs, between the MPS-selected line and the non-selected line (Fig. 5).

Growth hormone analysis

In experiment 1, the decrease in the concentration of GH was significantly greater in the MPS-selected Landrace line than in the non-selected Landrace line after the first vaccination (day 0) (Shimazu *et al.* 2014). Similarly, in experiment 2, GH concentration significantly decreased in the MPS-selected Landrace line; however, it increased in the non-selected Landrace line on days 7 and 14 after the second vaccination (Fig. 6).

DISCUSSION

Previous reports have clarified the immune responses of the MPS-selected Landrace line and the non-selected line to SRBCs (Katayama *et al.* 2011) and *M. hyopneumoniae* vaccination (Shimazu *et al.* 2013, 2014). In the present study, to confirm the repeatability of the observed effects on immune response, we compared both MPS resistance and immune response to *M. hyopneumoniae* vaccination in the MPS-selected Landrace line with non-selected Landrace lines. These non-

selected Landrace lines used in two experiments were genetically different lines.

First, we observed a significant difference in MPS lung lesion scores between the MPS-selected Landrace line and two non-selected Landrace lines. As expected, lung lesion scores were significantly lower in the MPS-

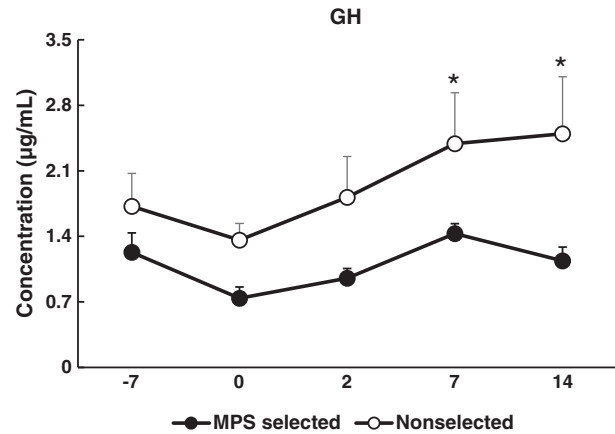


Figure 6 Changes in the concentrations of growth hormone (GH) in plasma samples obtained from mycoplasma pneumoniae of swine (MPS)-selected ● (closed circles) and non-selected ○ (open circles) Landrace pigs. Blood samples were collected on 5 days (–7, 0, 2, 7 and 14). MPS vaccination was conducted on those days –7 and 0 after blood sampling on those days. Statistical differences are indicated as the comparison between the two lines on the same day. Error bars indicate the standard error. (Asterisk mark indicates a significant difference at $P < 0.05$).

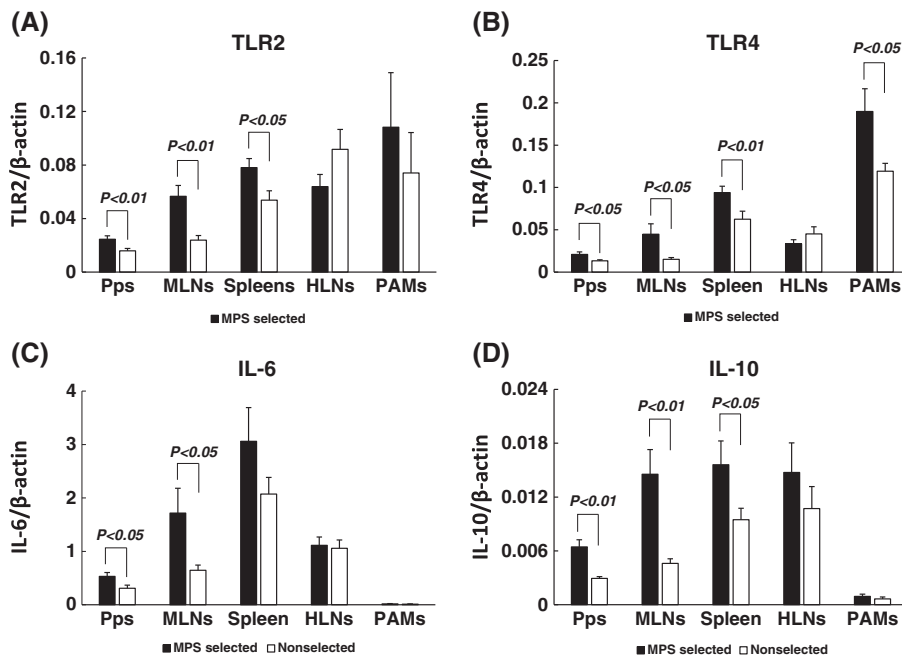


Figure 5 Mean and SE of the gene expression of toll-like receptors (TLRs) and cytokines in immune tissues from mycoplasma pneumoniae of swine (MPS)-selected (black bars) and non-selected Landrace (white bars) pigs. Pps, Peyer's patch, MLNs, mesenteric lymph nodes; HLNs, hilar lymph nodes; Spleens, spleens; and PAMs, pulmonary alveolar macrophages. The expression of TLR2 (A), TLR4 (B), interleukin (IL)-6 (C) and IL-10 (D) are shown as values of relative index (RI), which is calculated by dividing the expression levels of TLRs and cytokines by the expression levels of β -actin.

selected Landrace line than in the two non-selected Landrace lines. This result confirms the resistance to MPS of this selected line.

In experiments 1 and 2, various immune traits were measured. Therefore, we calculated the correlations between the MPS lung lesion and the each immune trait to clarify which immune traits were related to the MPS lung lesion. However, we could not get any significant correlations. Nevertheless, many significant differences in immune traits between the MPS-selected line and non-selected line were observed in both experiments.

From the results of experiment 1 (Shimazu *et al.* 2013, 2014) and 2, and previous results (Katayama *et al.* 2011), the possibility that the humoral immunity which is characterized by B cells and their activities were suppressed in the MPS-selected line against vaccination was suggested. Compared with the non-selected Landrace line, the percentage of B cells in peripheral blood (experiments 1 and 2) and their IgG secretions (experiment 2) and the proliferative abilities of PBMCs (experiment 1) were significantly decreased in the MPS-selected Landrace line. Furthermore, the same result was confirmed in our previous study that the SRBC-specific IgG production was decreased in the MPS-selected line (Katayama *et al.* 2011). In addition, previous studies have indicated that increased IgG production in serum is not directly involved in the clinical resistance to mycoplasma pneumonia (Thacker *et al.* 1998; Chen *et al.* 2006). These results also indicate that increased antibody-specific IgG production most likely contributes to MPS lung lesion severity during natural infection.

In association with the suppression of IgG in the MPS-selected Landrace line, the secretion of GH in peripheral blood was significantly suppressed in experiment 2 and experiment 1 (Shimazu *et al.* 2014). B cells are the major immune cells that express both GH and GH receptors (Hattori *et al.* 2001; Hattori 2009). GH promotes lymphocyte survival and proliferation (Jeay *et al.* 2002; Borghetti *et al.* 2006). Therefore, the decrease in B cell percentage, GH secretion and proliferative ability of PBMCs in the MPS-selected line suggests that these traits correlate with each other. Donahue *et al.* (2006) had reported that the secretion of GH was increased in male rats and female rats during diestrus exposed to a stressor. From this, the MPS-selected Landrace line may be less susceptible to stress than the non-selected Landrace line. However, whether the increase and decrease of GH is involved in MPS resistance is unclear.

Contrary to the decrease in the B cells and their IgG secretion, the salivary IgA secretion was increased in the MPS-selected Landrace line. Peripheral blood IgG and saliva IgA are produced by different tissues or organs. The IgG and monomeric type of IgA are known to be produced in lymph node, spleen and bone marrow, and then secreted in blood. The dimeric type of IgA secreted in saliva is mainly produced in submucous-lymph tissues, and then secreted in tears, saliva, sweat,

colostrum (Parham 2009). The mucosal immune system is a first defense line of inner body surfaces and IgA is essential in this surface defense system. Secretion of IgA is critical for protection against infectious pathogens (Phalipon *et al.* 2002; Brandtzaeg 2003; Tlaskalová-Hogenová *et al.* 2004). Increased IgA secretion may also play a protective role against *M. hyopneumoniae* infections in the upper respiratory tract (Woolard *et al.* 2004). The activation of IgA-dependent mucosal immunity may be one of the reasons the lung lesions were reduced in the MPS-selected line.

On the other hand, natural immunity and cell-mediated immunity, which are characterized by granular leukocyte percentage and INF- γ protein and mRNA expressions, showed significantly higher in the MPS-selected Landrace line than that in two non-selected Landrace lines. Increased INF- γ expression eliminates bacteria and parasites by activating macrophages and killer T cells (Romagnani 1992; Thacker *et al.* 2000; Jones *et al.* 2002; Woolard *et al.* 2004), and also plays an important role in pulmonary defense mechanisms against mycoplasma respiratory disease (Woolard *et al.* 2004; Rodriguez *et al.* 2007). Furthermore, cell-mediated immunity is important for controlling MPS (Thacker *et al.* 2000; Chen *et al.* 2006) and may be one of the reasons for the increased resistance to MPS in the MPS-selected line.

In the present study (experiment 2), the mRNA expressions of TLR2, TLR4, IL-6 and IL-10 of the Pps, MLNs, spleens and PAMs were significantly higher in those immune tissues obtained from the MPS-selected line than that of the non-selected line. These differences in immune response suggest a genetically correlated response according to MPS selection. By recognizing microbe-associated molecular patterns, the TLR family plays a critical role in host defense and in the development of inflammation. TLR2 recognizes ligands from Gram-positive bacteria, whereas TLR4 responds to endotoxin from Gram-negative bacteria. TLRs are also involved in the responses to lung injury (Jiang *et al.* 2005) and to *M. hyopneumoniae* infection (Chu *et al.* 2005; Wu *et al.* 2008; Love *et al.* 2010). IL-6 is a primary cytokine responsible for acute inflammation (Thacker 2006) and *M. hyopneumoniae* clearance in lungs (Pietsch *et al.* 1994; Wu *et al.* 2008). IL-10 is an endogenous regulator that is critical for chemokine expression in acute lung inflammation (Shanley *et al.* 2000). These results indicate that interactions between these cytokines and TLRs may contribute to the balance of the inflammatory and immune responses in the lungs of the MPS-selected line.

In conclusion, the MPS-selected Landrace line shows strong resistance against MPS due to the strength of its cell-mediated and mucosal immune responses, as described above. Furthermore, our research indicates that immune system components such as INF- γ , TLR2, TLR4 and salivary IgA may be working synergistically against MPS. In present studies, cytokine and TLR levels

in mucosal tissues, PAMs and salivary IgA were measured only in experiment 2. Therefore, it is necessary to confirm the repeatability of these results. In addition, although the MPS-selected Landrace line showed a strong resistance against MPS, further studies to confirm whether this MPS-selected line has resistance to other respiratory chronic diseases are warranted.

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